REMARKS

Claims 1-5 and 11-15 are currently pending. Claims 1, 3, 11, and 15 are amended herein to clarify aspects of the invention. Accordingly, instant claims 1-5 and 11-15 are under consideration.

Support for the amendments to the claims is found throughout the specification and in the original claims. More specifically, support for amendment to claim 1 is found, for example, in original claim 1 and in the specification at 5, line 30 through to page 6, line 2 and at page 6, lines 10-12 and 16-19 and the Sequence Listing. Support for amendment to claims 3 and 15 is found, for example, in original claims 3 and 15. Support for amendment to claim 11 is found, for example, in original claim 11 and in the specification at page 6, lines 16-19 and the Sequence Listing. No issue of new matter is introduced by the amendments to the claims.

Rejections under 35 U.S.C. § 112

Claims 1-5 and 11-15 are rejected under 35 U.S.C. § 112, first paragraph, for containing subject matter which was allegedly not enabled by the specification. In view of the clarifying amendments to the claims and Applicant's arguments presented herein, the rejection, as it applied to claims 1-5 and 11-15 is traversed.

The Office Action acknowledges that the specification is enabling for a method of treating a bronchoconstrictive disease, ARDS, by administering EV131 comprising the amino acid sequence of SEQ ID NO: 6, but maintains that the specification is allegedly not enabling for a method as recited in the claims.

The claims are amended to be directed to a method of treating a bronchoconstrictive disease mediated by neutrophil cells in a patient, comprising administering a histamine binding compound to the patient in a therapeutically-effective amount, wherein the histamine binding compound is selected from: a) EV131 protein comprising the amino acid sequence of SEQ ID NO: 6; or b) a fragment of the EV131 protein comprising the amino acid sequence of SEQ ID NO: 6 that retains a biological function of EV131, wherein the fragment comprises the sequence motif aspartic acid (D)/glutamic acid (E), alanine (A), tryptophan (W), and lysine (K)/arginine (R) and the

sequence motif tyrosine (Y)/cysteine (C), glutamic acid (E)/aspartic acid (D), leucine (L)/isoleucine (I)/phenylalanine (F), and tryptophan (W), and wherein said biological function is the ability to bind specifically to histamine with a dissociation constant of less than 10^{-7}M (claim 1) or a method of treating a bronchoconstrictive disease mediated by neutrophil cells in a patient, comprising administering a histamine binding compound to the patient in a therapeutically-effective amount, wherein the histamine binding compound is EV131 protein comprising the amino acid sequence of SEQ ID NO: 6. Accordingly, the instant claims provide distinguishing information concerning the EV131 protein and fragments thereof encompassed by the claims. Fragments comprising these conserved motifs are described in the specification as filed and one of skill in the art would be aware of the existence and functional significance of conserved regions in a number of histamine binding proteins as described in the art prior to the filing date of the present invention. More particularly, an ordinarily skilled practitioner in the field would be aware of documents such as WO 99/27104, of which Figure 22 depicts an alignment of various histamine binding proteins showing that the DAWK motif is highly conserved among these proteins. In light of the above, an ordinarily skilled practitioner would appreciate that fragments comprising the recited conserved motifs would confer upon such a fragment the recited functional properties.

With regard to the issues raised in connection with the treatment of bronchoconstrictive diseases, Applicant asserts that the inventor was the first person to demonstrate that the removal of histamine from a disease site using EV131 counteracts neutrophil-mediated diseases. Indeed, the effect of histamine depletion on neutrophil-mediated diseases had not been identified prior to the disclosure of the present invention. Quite to the contrary, previous attempts to explore the influence of histamine on neutrophil-mediated diseases suggested only a marginal effect of histamine on diseases within this category. As described in the specification at page 2, lines 22-32, the failure of these previous studies to appreciate the significant role of histamine in neutrophil-mediated diseases was likely due to the experimental design of these earlier studies, which targeted histamine receptors rather than histamine.

Given that the present inventor has demonstrated for the first time a pivotal role of histamine in neutrophil-mediated diseases, an ordinarily skilled practitioner would

appreciate that other neutrophil-mediated diseases would benefit from treatment using EV131 to remove histamine from relevant disease sites. Moreover, such a practitioner would realize, based on his/her experience, which neutrophil-mediated diseases would be most likely to respond to EV131, what the relevant sites involved in such diseases are, and how to administer EV131 thereto. Additional details pertaining to pharmaceutical compositions and administration thereof are presented in the specification at page 7, line 6 through to page 9, line 3. In support of Applicant's arguments, a number of documents published after the priority date of the present application show that EV131 positively influences allergic asthma and adult respiratory distress syndrome (ARDS). Exemplary of such documents, a paper by Couillin et al. (2004, J. Immunol. 173:3281-3286) and an abstract by Couillin et al. (2005, Ann. NY Acad. Sci. 1056:197-205) are submitted herewith for the Examiner's consideration as Exhibits A and B, respectively.

In light of the above, Applicant submits that the instant claims are enabled by the specification. Accordingly, reconsideration and withdrawal of the rejection of claims 1-5 and 11-15 under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 1-5 and 11-15 are rejected under 35 U.S.C. § 112, second paragraph, for alleged indefiniteness. In view of amendments to the claims, the rejection, as it applied to claims 1-5 and 11-15 is respectfully traversed.

In view of the above, Applicant submits that the language of the instant claims is definite. Accordingly, reconsideration and withdrawal of the rejection of claims 1-5 and 11-15 under 35 U.S.C. § 112, second paragraph, is respectfully requested.

Rejections under 35 USC § 102

Claims 1-2, 4-5, and 11-14 are rejected under § 102(b) as allegedly anticipated by WO 96/38481. A teleconference with the Examiner confirmed that citation of WO 96/38481 reflected a clerical error and it was the Examiner's intention to cite WO 99/27104. In view of Applicant's arguments presented herein, this rejection is respectfully traversed.

For the record, Applicant asserts that WO 99/27104 only discloses the use of EV131 as an anti-inflammatory agent or as an agent useful for counteracting the effects of allergic reactions. This reference fails to mention neutrophil-mediated diseases and,

furthermore, fails to teach or recite treatment of neutrophil-mediated diseases with antihistamine agents. That being the case, WO 99/27104 fails to teach or suggest at least one recited element of the claims.

In view of the above, reconsideration and withdrawal of the rejection of claims 1-2, 4-5, and 11-14 under § 102(b) is respectfully requested.

Rejections under 35 USC § 103

Claims 1-5 and 11-15 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over WO 99/27104. In view of Applicant's arguments presented herein, the rejection, as it applied to claims 1-5 and 11-15, is respectfully traversed.

As indicated above, WO 99/27104 fails to mention neutrophil-mediated diseases. The reference, furthermore, fails to mention ARDS, infant respiratory distress syndrome (IRDS), severe acute respiratory syndrome (SARS), chronic obstructive airways disease (COPD), cystic fibrosis, and ventilator induced lung injury (VILI) as recited in claim 3. In light of the above, WO 99/27104 fails to teach or suggest several recited elements of the claims. The Office Action refers to page 1, lines 16-21 for support that histamine regulates inflammatory processes. A review of this passage reveals that it provides only generic teaching pertaining to the effects of histamine. That being the case, WO 99/27104 fails to render obvious the present claims.

Moreover, as stated in the present specification, certain antihistamine agents have been used in the prior art to evaluate their utility in treating neutrophil-mediated conditions. See, for example, page 1, line 31 through to page 2, line 10 and page 2, lines 22-32. Indeed, all of the agents tested were of limited use. The failure of these antihistamine agents to influence the tested conditions would mean that, at the priority date of the present application, a person of ordinary skill in the art would not have expected the histamine binding proteins of the invention to be useful in treating neutrophil-mediated conditions even though the prior art teaches the use of histamine binding proteins for treating allergic, inflammatory, and autoimmune diseases. In short, the prior art teaches away from the present invention because it indicates that the claimed method would have, at best, limited expectation for success.

Furthermore, as stated on page 2, lines 22-32 of the specification, the role of histamine in neutrophil-mediated diseases was overlooked in the prior art because the anti-histamine compounds known in the prior art act on one or more of the histamine receptors. In contrast, the compounds used in the methods of the present invention bind to histamine itself. The inventor's discovery that targeting the histamine molecule directly could counteract neutrophil-mediated conditions was not known or suggested by the prior art. In sum, the link between histamine release and diseases mediated by neutrophils is not disclosed in WO 99/27104. In light of the above, Applicant asserts that this reference fails render obvious the method of instant claims.

In view of the above, Applicant deferentially requests that the rejection of claims 1-5 and 11-15 under 35 U.S.C. § 103(a) as allegedly unpatentable over WO 99/27104 be reconsidered and withdrawn.

Fees

No additional fees are believed to be necessitated by this amendment. However, should this be an error, authorization is hereby given to charge Deposit Account No. 11-1153 for any underpayment or to credit any overpayment.

Conclusion

It is submitted, therefore, that the claims are in condition for allowance. No new matter has been introduced. From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order, and such action is earnestly solicited. In the event that there are any questions concerning this amendment, or application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Respectfully submitted,

Sarah J. Fashena, Ph.D.
Agent for Applicant(s)
Registration No. 57,600

KLAUBER & JACKSON
411 Hackensack Avenue
Hackensack, New Jersey 07601
(201) 487-5800

November 2, 2009

Enclosures: Petition for a Two Month Extension of Time
Exhibit A: Couillin et al. (2004, J. Immunol. 173:3281-3286)
Exhibit B: Couillin et al. (2005, Ann. NY Acad. Sci. 1056:197-205; Abstract only)

EXHIBIT A

Arthropod-Derived Histamine-Binding Protein Prevents Murine Allergic Asthma

Isabelle Couillin,*† Isabelle Maillet,*† B. Boris Vargaftig,¶ Muazzam Jacobs,‖ Guido C. Paesen,§ Patricia A. Nuttall,§ Jean Lefort,¶ René Moser,‖ Wynne Weston-Davies,‡ and Bernhard Ryffel[1]*

Because histamine receptor type I blockade attenuates allergic asthma, we asked whether complete neutralization of histamine by an arthropod-derived, high affinity histamine-binding protein (EV131) would prevent allergic asthma. Intranasal administration of EV131 given before Ag challenge in immunized mice prevented airway hyperreactivity by 70%, and abrogated peribronchial inflammation, pulmonary eosinophilia, mucus hypersecretion, and IL-4 and IL-5 secretion. Saturation with histamine abrogated the inhibitory effect of EV131 on bronchial hyperreactivity. The inhibitory effect of EV131 on bronchial hyperreactivity was comparable to that of glucocorticosteroids. These results demonstrate that histamine is a critical mediator of allergic asthma. Therefore, complete neutralization of histamine, rather than specific histamine receptor blockade, may have a profound effect on allergic asthma. *The Journal of Immunology,* **2004, 173: 3281–3286.**

Allergic asthma is a chronic disease characterized by reversible obstruction of the airways, bronchial hyperresponsiveness (BHR),[2] edema, infiltration of lungs by inflammatory cells, and mucus overproduction (1–3). Allergen-specific Th2 lymphocytes producing IL-4, IL-5, IL-13, IgE, and mucus overproduction are the hallmarks of asthma. In asthmatic individuals, a second contact with the sensitizing allergen results in early and late inflammatory responses. The early reaction is due to IgE-mediated mast cell degranulation, with the release of preformed bioactive molecules such as histamine and 5-hydroxytryptamine, which induce mucus hypersecretion, bronchoconstriction, and increased protein exudation. Although the initial phase may resolve, it is followed by a late-phase response characterized by infiltration of eosinophils and lymphocytes, accompanied by BHR. The repeated exposure to allergens promotes chronic inflammation, leading to the long-term sequellae of asthma.

Histamine, first identified as a potent vasoactive amine, is now recognized for its multiple regulatory activities in the respiratory, digestive, and immune systems, and CNS. Mast cells and basophils are the major producers of preformed histamine that they release from intracellular granules in response to Ag-mediated cross-linking of IgE receptors. Nevertheless, other cells release neo-formed histamine immediately after its synthesis, such as neutrophil, platelets (4), dendritic cells (DCs) (5), and T cells (6), pointing to the important role of this molecule. In lungs, mast cells are present in bronchial walls near vessels, in muscles, and in the bronchial lumen (7). Histamine was one of the first inflammatory mediators of allergic asthma recognized in human and guinea pig models (8). Interestingly, histamine has been shown to modulate cytokine production in different cell types (9), Ab and T cell responses (10) maintaining a predominant Th2 response in allergic disorders (11). Histamine exerts its effects through four receptor subtypes: histamine receptor 1 (HR1) and HR2, both expressed on lymphoid and nonlymphoid cells; HR3, mainly expressed in the brain (12); and HR4, which has been described recently in leukocytes (13), but is also present in airways (14). However, the role of histamine as a critical effector molecule in a murine model of allergen-induced bronchoconstriction has been questioned, because histamine itself fails to induce direct bronchoconstriction under conditions in which 5-hydroxytryptamine and acetylcholine are effective (15). Furthermore, HR1 antagonists are not recognized as clinically effective therapy against asthma (16)

Although blood-feeding ectoparasites can cause histamine-mediated inflammation in the host, ticks have evolved to suppress inflammation and facilitate feeding by secreting histamine-binding proteins at the site of feeding (17–19). The tick *Rhipicephalus appendiculatus* secretes three histamine-binding proteins, which have been purified, cloned, expressed, and characterized (20). Their crystallographic structure and biological activity indicate that they sequester histamine, competing with HRs for histamine binding. Both histamine-binding proteins rEV131 and rEV504 bind histamine with high affinity. However, rEV131 has a distinctive feature because it presents a second specific binding site for histamine with lower affinity than the high affinity binding site (20, 21).

In this study, using rEV131, we demonstrate that neutralization of histamine inhibits experimental allergic asthma, suggesting that histamine is a critical mediator in its pathogenesis, and opening new perspectives for asthma therapy.

Materials and Methods

Animals and immunization

The BP2 strain of mice was obtained from Janvier, France (15, 22). The local ethics committee approved all protocols used in this study. The mice, aged 6–8 wk, were immunized s.c. twice at weekly intervals with a 0.4 ml saline containing 100 μg of OVA and 1.6 mg of alum for the initial experiments, and for subsequent experiments the Ag dose was reduced to 1 μg of OVA per injection. One week after the second immunization, at day 14, intranasal challenge was performed under light i.v. ketamine anesthesia by applying 50 μl of OVA in alum-free saline solution (10 μg) or saline alone as a control.

*Centre National de la Recherche Scientifique, Institute Transgenose, Orleans, France; †Key-Obs S. A., Orleans, France; ‡Evolutec, Ltd., Oxford, United Kingdom; §Centre for Ecology and Hydrology, Oxford, United Kingdom; ¶Deptartment of Pharmacology, Instituto de Ciências Biomédicas, University of S. Paulo, Brazil; and ‖Institute for Biopharmaceutical Research, Matzingen, Switzerland

Received for publication April 17, 2003. Accepted for publication June 10, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Bernhard Ryffel, Institute Transgenose, Centre National de la Recherche Scientifique, 41500 Orleans, France. E-mail address: bryffel@cnrs-orleans.fr

[2] Abbreviations used in this paper: BHR, bronchial hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; HR, histamine receptor; Penh, enhanced respiratory pause.

0022-1767/04/$02.00

The rEV131 protein (340 μg, m.w. 20,406; Evolutec) or budesonide (375 μg, m.w. 430.5; AstraZeneca, Uppsala, Sweden), a potent corticosteroid (positive control), was administered intratracheally (50 μl in saline buffer) under ketamine anesthesia, 1 h before the Ag challenge and/or immediately before the OVA challenge. However, a single administration of EV131, 1 h prior to challenge, proved to be equally effective. We chose the intratracheal route for the administration of the protein and budesonide to assure optimal airways deposition, although the intranasal administration was active. To ascertain that the effect is due to histamine scavenging, histamine-saturated EV131 (incubation at molar ratios 1 and 2 for 30 min of histamine and EV131) was compared with native EV131 for inhibition of BHR (histamine dihydrochloride, m.w. 184; Sigma-Aldrich, St. Louis, MO). The HR antagonists, mepyramine (340 μg, m.w. 402; Sigma-Aldrich), cimetidine (340 μg, m.w. 409; Sigma-Aldrich), and thioperamide (340 μg, m.w. 409; Sigma-Aldrich) were administered intranasally in 50 μl with saline buffer 1 h before the Ag challenge.

Airways resistance

The airways resistance was evaluated by whole-body plethysmography (2). BHR to aerosolized methacholine was investigated at several time points after OVA challenge. Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronics, Sharon, CO). Mice were ventilated on high oxygen conditions to avoid hypoxemia induced by methacholine administration. Methacholine at 50 mM was aerosolized for 1 min, and mean airway bronchoconstriction readings, as assessed by enhanced respiratory pause (Penh), were obtained over 15-min periods. Penh can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves; increased phase shift correlates with increased respiratory system resistance.

Penh is calculated by the formula $\text{Penh} = (\text{Te/RT} - 1) \times \text{PEF/PIF}$, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow.

The mouse is placed in barometric plethysmography with two chambers linked to suction pump, which ensure constant airflow, as described before. The animal is introduced in the first chamber separated from the second in which pressure corresponds to atmospheric pressure. Every compartment is linked to two parts of a differential pressure captor, connected to an electronic amplifier, and signals are analyzed by software. This system allows quantification of many parameters during several respiratory cycles. Penh values correspond to means of 11 events (cycles) every 5 s in raw data. Penh values are indicated for three points before (−3 to −1 min) and 14 points after (+1 to +14 min) methacholine nebulization. In this case, indicated Penh value is the mean of Penh values between 1 min before and 1 min after the point (e.g., Penh values at +5 min correspond to the mean of all values between point +4 min and point +6 min).

Bronchoalveolar lavage (BAL)

BAL was performed 3 days after intranasal challenge by canulating the trachea under ketamine anesthesia and washing four times with 0.5 ml each of ice-cold PBS. The lavage fluid was centrifuged, and the supernatant was frozen for cytokine determinations. The cell pellet was resuspended in PBS and counted by a hemocytometer chamber, and cytospin preparations were made using a Shandon cytocentrifuge. The cells were analyzed after differential staining with May-Grüenwald-Giemsa.

Lung histology

After BAL, the mice were killed (3 days after OVA challenge). The whole lung was removed and fixed in 4% buffered formaldehyde for standard microscopic analysis H&E and periodic acid Schiff reagent staining. The peribronchial infiltrate and the smooth muscle hyperplasia were assessed by a semiquantitative score (0–3) by two independent observers.

Dosage of IL-4 and IL-5

IL-4 and IL-5 in the BAL fluid were evaluated by ELISA. Briefly, 96-well microtiter plates were coated with monoclonal rat anti-mouse IL-4 (BVD4-1D11; BioSource International, Camarillo, CA) or monoclonal rat anti-mouse IL-5 (R&D Systems, Lille, France) in 0.1 M carbonate buffer, pH 8.2, and incubated overnight at 4°C. Plates were washed and blocked with PBS containing 1% of BSA for 2 h. After washing, dilutions of recombinant murine IL-4 or IL-5 or samples were applied overnight at 4°C. Then biotinylated rat anti-IL-4 Ab (BVD6-24G2; BioSource International) or biotinylated rat anti-IL-5 Ab (R&D Systems) was added for 2 h at 4°C. Plates were washed and incubated with ExtrAvidin peroxidase conjugate (at 0.05 μg/ml to each well; Sigma-Aldrich) for 45 min at room temperature. Plates were developed with tetramethylbenzidine substrate (Dynex, Cergy-Pontoise, France). The reaction was stopped with sulfuric acid 2 N, and the plates were read at 450 nm with an automatic microplate reader. The lower limits of detection of these assays are ~10 pg of IL-4/ml sample and 5 pg of IL-5/ml.

Statistical analysis

Data are presented as means and SEM indicated by error bars. Statistical significance was determined by Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

The histamine-binding protein EV131 prevents BHR in Ag-sensitized mice

Because histamine is produced during allergic asthma, we tested the potential interference of the arthropod-derived histamine-binding protein EV131 on BHR. Upon Ag challenge, the mice developed a robust BHR in response to aerosolized methacholine at 24 h (Fig. 1*A*). Administration of EV131 by the intratracheal route, 1 h and just before OVA challenge, inhibited methacholine-induced bronchoconstriction (70% of controls; $p < 0.01$). The vehicle alone, saline, had no effect on BHR. The effect of EV131 on BHR was matched by the inhibition induced by glucocorticosteroid budesonide, used as a control inhibitor (70%; $p < 0.01$). To test whether the inhibitory effect is related to histamine scavenging, we compared histamine-presaturated EV131 and native EV131 on BHR. As expected, the inhibitory effect of presaturated EV131 with a 2-molar excess of histamine was abrogated (Fig. 1*B*). EV131 presaturated at equimolar histamine showed loss of activity as compared with native EV131 on BHR of OVA-challenged mice, suggesting that the two histamine pockets are implicated in histamine scavenging.

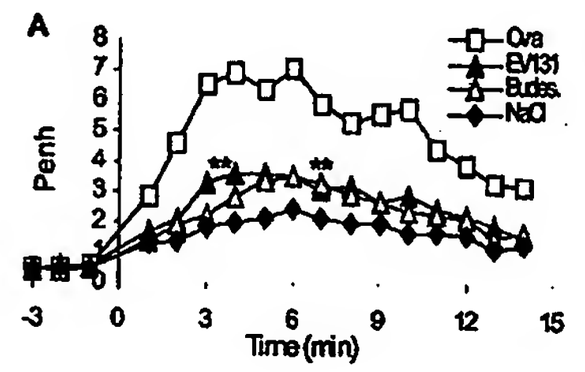

FIGURE 1. EV131 inhibits OVA-induced BHR in mice sensitized with Ag (*A*), while histamine-saturated EV131 loses its activity (*B*). *A*, BP2 mice were immunized on days 0 and 7 with OVA. The mice were given 50 μl of NaCl (saline) or EV131 (340 μg in NaCl) intratracheally and challenged with 50 μl of NaCl or OVA 1 h later. BHR to methacholine was measured 24 h after challenge by the noninvasive enhanced pause (Penh) and expressed as a function of time. The results represent the mean for two independent experiments ($n = 8$ mice per group). Inhibition by EV131 and by budesonide is significant at $p < 0.01$ (**). *B*, Histamine-saturated EV131 (molar ratio 1 and 2) and native EV131 were given to immunized mice in the identical protocol as described before. Histamine-saturated EV131 (molar ratio 2) lost its inhibitory effect and was significantly different from native EV131 ($p < 0.01$), while EV131 with histamine at equimolar amounts had partial activity ($p < 0.05$). Representative results from two independent experiments ($n = 4$ mice per group).

Therefore, in situ neutralization of histamine by EV131 acting as a soluble receptor with high affinity binding for histamine had a profound effect on BHR, and we therefore asked whether the recruitment of eosinophils during the allergic asthma was influenced.

Reduction of pulmonary inflammation and of the recruitment of eosinophils by EV131

Ag challenge caused a substantial recruitment of inflammatory cells into the BAL fluid at 72 h. Administration of EV131 before OVA challenge reduced significantly the numbers of mononuclear cells in the BAL fluid ($p < 0.05$; Fig. 2*A*). Budesonide had a slightly more pronounced effect ($p < 0.01$; Fig. 2*A*). Only few eosinophils and neutrophils were found in the BAL fluid of saline-challenged mice. By contrast, the Ag challenge resulted in a significant increase of eosinophil counts in the BAL fluid ($p < 0.01$; Fig. 2*B*). EV131 administration before the Ag challenge prevented largely the recruitment of eosinophils ($p < 0.01$; Fig. 2*B*). Furthermore, we demonstrated that the prevention of eosinophil recruitment was due to histamine scavenging, as histamine-presaturated EV131 was ineffective (data not shown). Finally, budesonide had an inhibitory effect on the recruitment of all cell types ($p < 0.01$; Fig. 2*B*). Representative cytospin preparations of BAL obtained from saline control or OVA-challenged mice in the presence or absence of inhibitors are shown in Fig. 3.

We further investigated the recruitment of eosinophils and other inflammatory cells in lung tissue sections from OVA-sensitized and challenged mice. OVA challenge in immunized mice caused peribronchial cell recruitment and hyperplasia of the bronchial smooth muscle and of goblet cells containing mucus (Fig. 4). Administration of EV131 reduced significantly the peribronchial eosinophilia, mucus hypersecretion, and hyperplasia of bronchial smooth muscles (Fig. 4). Therefore, complete in vivo neutralization of histamine with the high affinity histamine-binding protein

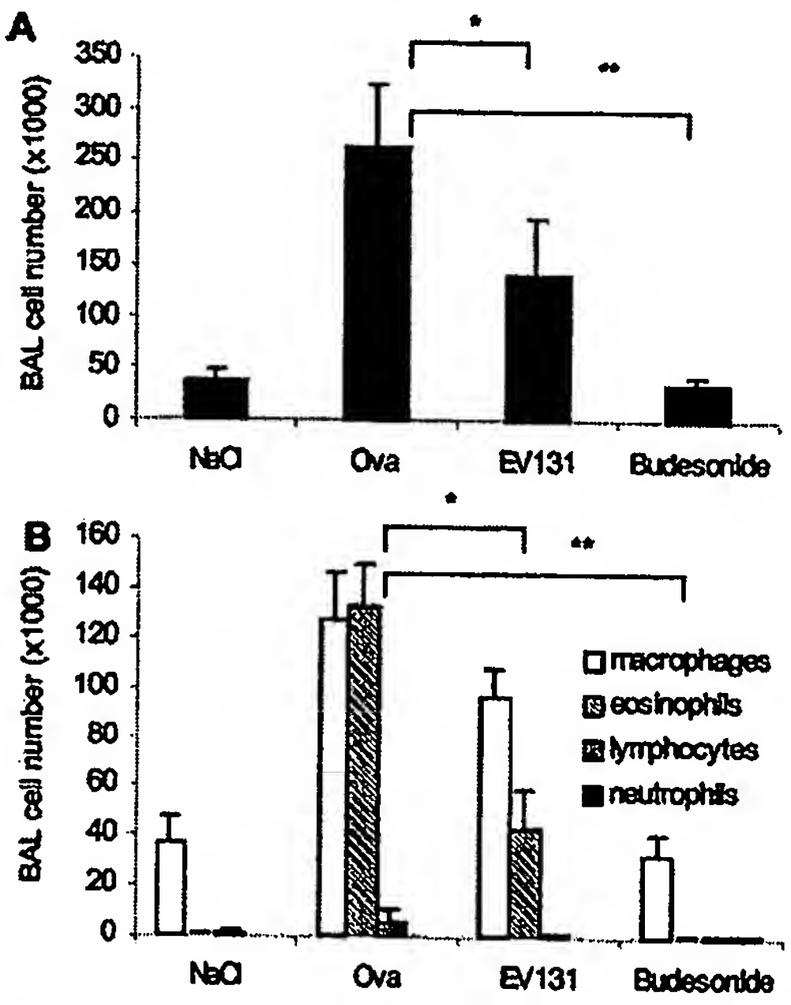

FIGURE 2. EV131 inhibits OVA-induced eosinophil recruitment in BAL fluid. BAL fluid was collected at 72 h after OVA challenge, and cytospin preparations were prepared: Total cell counts (*A*) and differential cell counts in BAL (*B*). The results are pooled from two independent experiments; mean values and SEM are given ($n = 8$ mice per group). Differences are significant, $p < 0.05$ (*) and $p < 0.01$ (**), respectively.

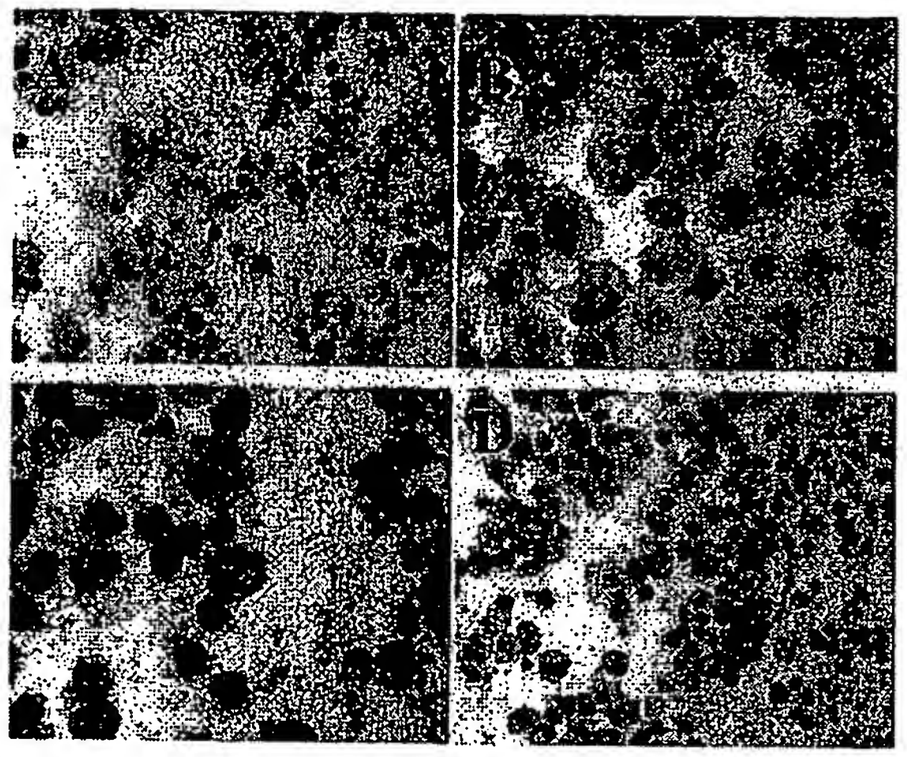

FIGURE 3. Cytospin preparation of BAL with reduced eosinophil recruitment by EV131. Micrographs of cytospin preparations of BAL at 72 h after NaCl (*A*) or OVA (*B*) challenge compared with OVA-challenged mice administered EV131 (*C*) or budesonide (*D*). May Grüenwald Giemsa staining and magnification ×100. Arrows mark eosinophils in cytospin preparations. The micrographs are representatives for three independent experiments (four mice per group per experiment).

EV131 inhibited the inflammatory cell recruitment and suppressed the characteristic allergic inflammation of the airways.

EV131 reduced IL-4 and IL-5 levels in BAL fluid

Allergic asthma involves the recruitment of Th2-biased T cells, resulting in increased production of Th2 cytokines. Ag challenge of immunized mice resulted in increased levels of IL-4 and levels of IL-5 (Fig. 5; $p < 0.01$) in the BAL fluid. EV131 given before Ag challenge inhibited significantly IL-4 and IL-5 (Fig. 5; $p < 0.05$) levels. The steroid control budesonide was slightly more effective than EV131 in inhibiting the production of IL-4 and of IL-5 ($p < 0.01$). Therefore, neutralization of histamine with EV131 appears to inhibit the allergen-induced Th2 cytokine response.

Duration of the inhibitory effect of EV131 on BHR and comparison with HR blockade

We further investigated the duration of the effect of EV131 on BHR. The inhibitory effect of EV131 was maintained for 48 h after OVA challenge in all mice (Fig. 6*A*) and, although reduced, was still present at 72 h, when animals received two intratracheal administrations of EV131 (Fig. 6*B*). Thus, EV131 administered before Ag challenge has a persistent effect.

We finally studied the efficacy of classical HR antagonists in the murine model of allergic asthma. Mepyramine, a selective HR1 antagonist and thioperamide, with mixed HR3 and HR4 antagonistic activity, reduced BHR (Fig. 7). Cimetidine, a classical HR2 antagonist, had no effect on BHR. Therefore, HR1, HR3, and possibly HR4 antagonists have inhibitory effects on BHR, but full inhibition requires on a molar basis larger amounts. Using EV131, which sequesters histamine, may be an interesting and effective alternative approach to inhibit BHR.

Discussion

We show in this study that the high affinity histamine-binding protein EV131 prevents experimental allergic asthma, supporting a critical role for histamine in Ag-induced BHR and bronchial inflammation. It is reasonable to assume that EV131 by locally sequestrating histamine prevents the access to its receptors.

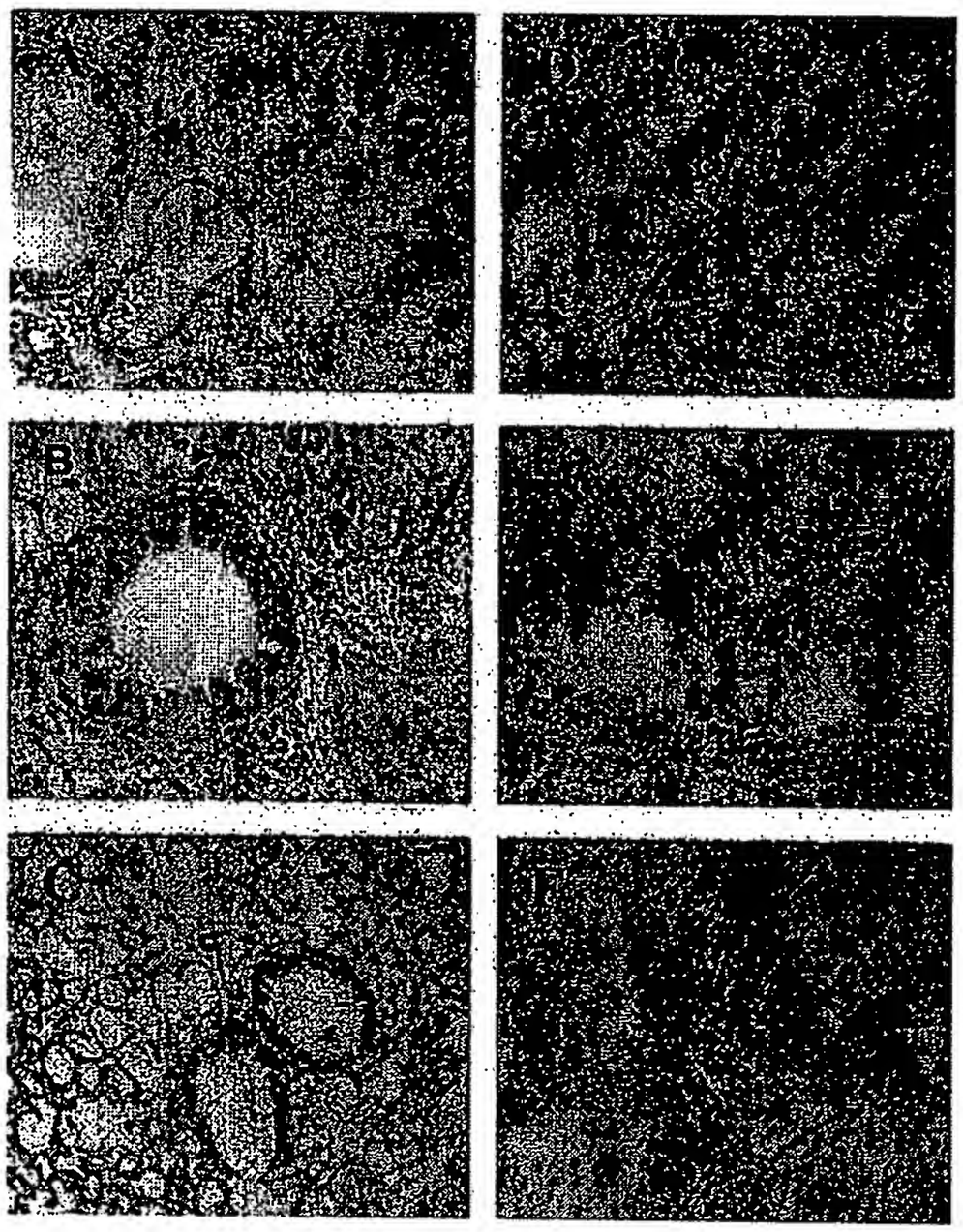

FIGURE 4. EV131 inhibits inflammation and mucus hypersecretion in OVA-challenged mice. Inflammatory changes of lungs of saline (*A*)- and OVA (*B*)-challenged mice: significant peribronchial and perivascular eosinophil infiltration in mice challenged with OVA (arrows mark peribronchial infiltration by eosinophils). EV131 inhibits peribronchial and perivascular eosinophil infiltration (*C*). H&E staining. EV131 inhibits mucus secretion of lungs in OVA-challenged mice. Mucus secretion changes in lungs of saline (*D*)- and OVA (*E*)-challenged mice: significant mucus secretion in mice challenged with OVA (arrows mark mucus secreted by epithelial cells). EV131 inhibits mucus secretion in mice challenged with OVA (*F*). Periodic acid Schiff mucus staining. Intermediate power micrographs are shown on *A*, *C*, *D*, *E*, and *F* (magnification ×200), and a higher magnification shows eosinophil infiltration on *B* (magnification ×400). These micrographs are representatives of three independent experiments, with four mice per group each.

Histamine plays a critical role in allergic asthma because it induces contraction and hyperplasia of airway smooth muscles, mucus secretion, plasma exudation, and vasodilatation, essentially via HR1 and HR4 (12, 23). Modulation of BHR by HR antagonists in mice has already been reported (24). In agreement with previous observations, we confirm in our system that a high dose of HR1 antagonist mepyramine blocks BHR, whereas HR2 antagonist cimetidine does not share this property. High dose of thioperamide, which has HR3 and HR4 antagonistic properties, also inhibited BHR in the murine model. Despite sequence homology and structural similarities, HR3 and HR4 have distinct pattern of expression. Indeed, HR4 subtype is essentially expressed on eosinophils, DCs, basophils, Th cells, mast and B cells, and in human lung cells, including fibroblasts, smooth muscle, epithelial, and endothelial cells (13, 25–27), whereas HR3 subtype is mainly located in the brain (28). Prevention of BHR by thioperamide and the specific expression pattern of HR4 subtype suggest that stimulation of this receptor is involved in allergic disorders. It can be postulated that the profound effects induced by the histamine-binding protein EV131 are in part due to the fact that HR4 is not activated, in

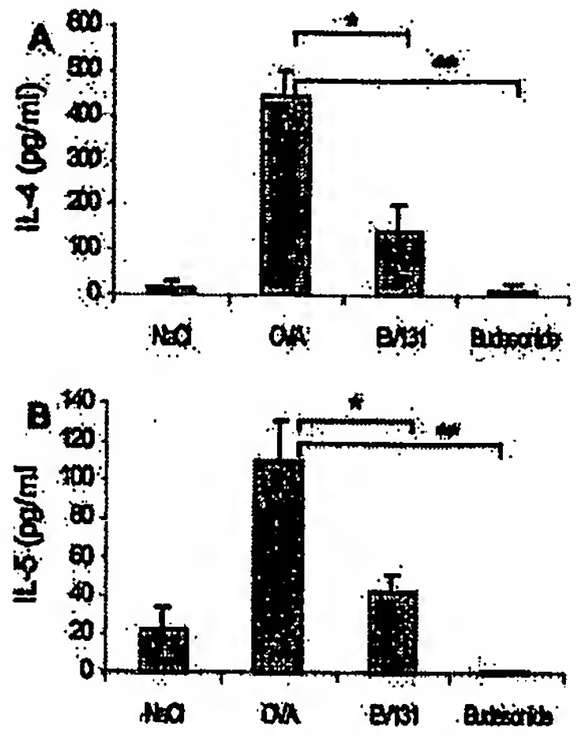

FIGURE 5. Reduced IL-4 and IL-5 in BAL fluid by EV131 administration. IL-4 (*A*) and IL-5 (*B*) levels were measured in the BAL fluid by ELISA 24 h after NaCl or OVA challenge. Significant increased IL-4 and IL-5 levels were found in mice challenged with OVA vs NaCl. EV131 inhibits IL-4 and IL-5 secretions in mice challenged with OVA. Mean values and SEM are given from two independent experiments (eight mice per group), $p < 0.05$ (*) and $p < 0.01$ (**).

addition to suppression of the other HR. The presently available HR antagonists are of limited clinical use, because the high doses required to inhibit allergic asthma lead to unacceptable adverse effects (29).

EV131 has a high affinity binding for histamine, as no competition was observed with other related compounds such as the HR1 antagonist mepyramine and the HR2 antagonist cimetidine in vitro assays (20). EV131 binds histamine in a competitive manner toward the HR1 on the smooth muscle cells of guinea pig ileum (20). Furthermore, EV131 inhibits histamine, but not serotonin- or bradykinin-induced contraction of guinea pig ileum. The effect of EV131 is dose dependent, and saturating concentrations of histamine or preincubation of EV131 with histamine abrogate the inhibitory effect of EV131 (G. Paesen, unpublished observations). To ascertain that the effect on the allergic lung model is due to

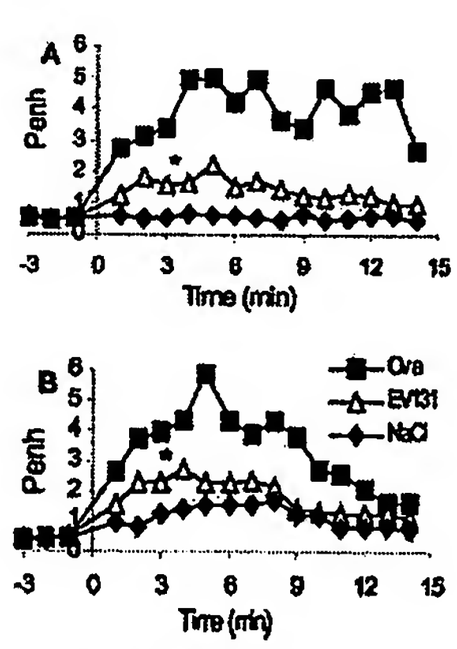

FIGURE 6. The inhibitory effect of EV131 on BHR is effective for 48 and 72 h after OVA challenge. OVA- compared with NaCl-challenged mice still exhibit BHR to methacholine at 48 h (*A*) and 72 h (*B*) postchallenge. EV131 (340 μg) administration still inhibits this BHR at 48 h (*A*) and at 72 h (*B*). Mean values with SEM are given, and the results are representative of two independent experiments ($n = 4$ mice per group). *, $p < 0.05$. Inhibition by EV131 at 48 h: $p < 0.01$ (**) for time 2 min, and thereafter $p < 0.05$ (*) (*A*). Inhibition by EV131 at 72 h: $p < 0.05$ (*) from 5 min onward (*B*).

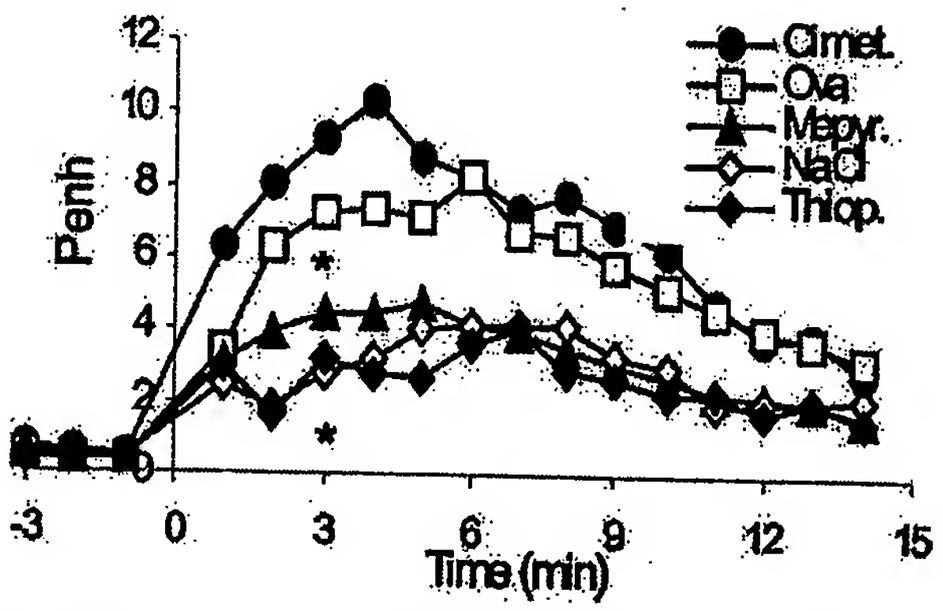

FIGURE 7. Inhibition of OVA-induced BHR by HR blockade. Inhibition of OVA-induced bronchial hypersensitivity by HR1 antagonist mepyramine and by HR2 antagonist thioperamide. Mice were treated with NaCl, mepyramine, cimetidine, or thioperamide in 50 μl of NaCl buffer 1 h before challenged with OVA. Penh values were measured 24 h after OVA challenge (n = 8 mice per group). Inhibition by mepyramine: $p < 0.01$ between 3 and 6 min, and thereafter $p < 0.05$ (*). Inhibition by thioperamide: $p < 0.01$ between 2 and 6 min, and thereafter $p < 0.05$ (*).

histamine scavenging, we tested presaturated EV131. We showed that the histamine-saturated EV131 lost its inhibitory activity on BHR and eosinophil recruitment.

These findings account for the ability of EV131 to sequester histamine. Indeed, the crystal structure of EV131 reveals two high affinity sites for histamine binding (20). Allergen challenge enhances histamine release upon OVA-specific IgE cross-linking on mast cell and subsequent degranulation with release of histamine in sensitized mice, leading to bronchoconstriction, eosinophilia, and mucus hypersecretion (30). Histamine may also induce maturation of DCs, which polarize naive $CD4^+$ T cells toward a Th2 phenotype, altering the repertoire of cytokines and chemokines secreted by mature DCs, as compared with DCs that have matured in the absence of histamine (11, 31, 32). Immature DCs express two active HRs, HR1 and HR2 (31). Histamine signaling through HR1 and HR2 increases IL-10 production and reduces IL-12 secretion (11, 33, 34). Therefore, local histamine neutralization by EV131 might abrogate in part DC differentiation and thereby contribute T cell polarization in the lung. EV131 reduced the allergen-induced recruitment of eosinophils as well as mucus hyperproduction, which may be due to the direct neutralizing effects of histamine or the modulation of the cytokine profile. Indeed, IL-4 and IL-5 levels in the BAL fluid of OVA-challenged mice were down-regulated by EV131. Moreover, as eosinophils are an important source of IL-4 in tissues, the reduced eosinophil recruitment observed following EV131 treatment may contribute to the diminution of IL-4 observed in the BAL fluid (35, 36). Conversely, IL-4 and IL-5 affect eosinophil recruitment and mucus production (37–39), and thereby may reduce the allergic response. IL-13 is also involved in the development of BHR and accumulation of eosinophils in the lungs, but we were unable to measure this Th-2 cytokine in the BAL fluid of OVA-challenged mice with our detection system (data not shown). Finally, we tested the effect of EV131 on exogenous histamine-induced BHR, and find that EV131 prevents BHR induced by intratracheally administered histamine (data not shown).

Antihistamines, essentially HR1 antagonists, have been used for decades against asthma, but with very limited efficacy, particularly as a monotherapy. Because the effects of histamine are mediated by at least four receptor subtypes, located in different cells and tissues, the neutralization by histamine-binding protein may be more efficient than an antagonism at the level of a specific receptor subtype. Furthermore, avoiding the blockade of receptors, such a therapeutic approach could trigger less adverse effects than antagonists and steroids. We show in this study that neutralizing histamine at the early phase may prevent the development of the late phase, and thereby control the asthmatic response.

In conclusion, topical neutralization of histamine in vivo with a high affinity arthropod-derived histamine-binding protein inhibits murine allergic asthma, suggesting a novel therapeutic approach that may be superior to the blockade of single HRs.

Acknowledgments

We gratefully acknowledge Drs. Valerie Quesniaux and Marie-Noëlle Soler for corrections and suggestions, and Thomas Robert for excellent technical assistance.

References

1. Kon, O. M., and A. B. Kay. 1999. T cells and chronic asthma. *Int. Arch. Allergy Immunol. 118:133.*
2. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol. 17:255.*
3. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science 282:2258.*
4. Saxena, S. P., L. J. Brandes, A. B. Becker, K. J. Simons, F. S. LaBella, and J. M. Gerrard. 1989. Histamine is an intracellular messenger mediating platelet aggregation. *Science 243:1596.*
5. Szeberenyi, J. B., V. Laszlo, E. Pallinger, E. Orso, G. Rothe, G. Schmitz, and A. Falus. 2001. Intracellular histamine content increases during in vitro dendritic cell differentiation. *Inflamm. Res. 50 (Suppl. 2):S112.*
6. Kubo, Y., and K. Nakano. 1999. Regulation of histamine synthesis in mouse $CD4^+$ and $CD8^+$ T lymphocytes. *Inflamm. Res. 48:149.*
7. Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev. 77:1033.*
8. Tohda, Y., H. Kubo, R. Haraguchi, T. Iwanaga, M. Fukuoka, and S. Nakajima. 1998. Roles of histamine receptor in a guinea pig asthma model. *Int. J. Immunopharmacol. 20:565.*
9. Bachert, C. 1998. Histamine: a major role in allergy? *Clin. Exp. Allergy 28 (Suppl. 6):15.*
10. Jutel, M., S. Klunker, M. Akdis, J. Malolepszy, O. A. Thomet, T. Zak-Nejmark, K. Blaser, and C. A. Akdis. 2001. Histamine up-regulates Th1 and down-regulates Th2 responses due to different patterns of surface histamine 1 and 2 receptor expression. *Int. Arch. Allergy Immunol. 124:190.*
11. Caron, G., Y. Delneste, E. Roelandts, C. Duez, J. Y. Bonnefoy, J. Pestel, and P. Jeannin. 2001. Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. *J. Immunol. 167:3682.*
12. Schneider, E., M. Rolli-Derkinderen, M. Arock, and M. Dy. 2002. Trends in histamine research: new functions during immune responses and hematopoiesis. *Trends Immunol. 23:255.*
13. Oda, T., N. Morikawa, Y. Saito, Y. Masuho, and S. Matsumoto. 2000. Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J. Biol. Chem. 275:36781.*
14. Coge, F., S. P. Guenin, H. Rique, J. A. Boutin, and J. P. Galizzi. 2001. Structure and expression of the human histamine H4-receptor gene. *Biochem. Biophys. Res. Commun. 284:301.*
15. Eum, S. Y., X. Norel, J. Lefort, C. Labat, B. B. Vargaftig, and C. Brink. 1999. Anaphylactic bronchoconstriction in BP2 mice: interactions between serotonin and acetylcholine. *Br. J. Pharmacol. 126:312.*
16. Nelson, H. S. 2001. Combination therapy of bronchial asthma. *Allergy Asthma Proc. 22:217.*
17. Ribeiro, J. M., and F. A. Walker. 1994. High affinity histamine-binding and antihistaminic activity of the salivary nitric oxide-carrying heme protein (nitrophorin) of *Rhodnius prolixus*. *J. Exp. Med. 180:2251.*
18. Weichsel, A., J. F. Andersen, D. E. Champagne, F. A. Walker, and W. R. Montfort. 1998. Crystal structures of a nitric oxide transport protein from a blood-sucking insect. *Nat. Struct. Biol. 5:304.*
19. Chinery, W. A., and E. Ayitey-Smith. 1977. Histamine blocking agent in the salivary gland homogenate of the tick *Rhipicephalus sanguineus sanguineus*. *Nature 265:366.*
20. Paesen, G. C., P. L. Adams, K. Harlos, P. A. Nuttall, and D. I. Stuart. 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. *Mol. Cell 3:661.*
21. Paesen, G. C., P. L. Adams, P. A. Nuttall, and D. L. Stuart. 2000. Tick histamine-binding proteins: lipocalins with a second binding cavity. *Biochim. Biophys. Acta 1482:92.*
22. Eum, S. Y., S. Haile, J. Lefort, M. Huerre, and B. B. Vargaftig. 1995. Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin 5-dependent bronchial hyperresponsiveness. *Proc. Natl. Acad. Sci. USA 92:12290.*
23. White, J. R., D. Zembryki, N. Hanna, and S. Mong. 1990. Differential inhibition of histamine release from mast cells by protein kinase C inhibitors: staurosporine and K-252a. *Biochem. Pharmacol. 40:447.*

24. De Bie, J. J., P. A. Henricks, W. W. Cruikshank, G. Hofman, E. H. Jonker, F. P. Nijkamp, and A. J. Van Oosterhout. 1998. Modulation of airway hyperresponsiveness and eosinophilia by selective histamine and 5-HT receptor antagonists in a mouse model of allergic asthma. *Br. J. Pharmacol. 124:857.*
25. Nguyen, T., D. A. Shapiro, S. R. George, V. Setola, D. K. Lee, R. Cheng, L. Rauser, S. P. Lee, K. R. Lynch, B. L. Roth, and B. F. O'Dowd. 2001. Discovery of a novel member of the histamine receptor family. *Mol. Pharmacol. 59:427.*
26. Morse, K. L., J. Behan, T. M. Laz, R. E. West, Jr., S. A. Greenfeder, J. C. Anthes, S. Umland, Y. Wan, R. W. Hipkin, W. Gonsiorek, et al. 2001. Cloning and characterization of a novel human histamine receptor. *J. Pharmacol. Exp. Ther. 296:1058.*
27. Liu, C., X. Ma, X. Jiang, S. J. Wilson, C. L. Hofstra, J. Blevitt, J. Pyati, X. Li, W. Chai, N. Carruthers, and T. W. Lovenberg. 2001. Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in bone marrow. *Mol. Pharmacol. 59:420.*
28. Yates, S. L., C. E. Tedford, R. Gregory, G. P. Pawlowski, M. K. Handley, D. L. Boyd, and L. B. Hough. 1999. Effects of selected histamine H3 receptor antagonists on tele-methylhistamine levels in rat cerebral cortex. *Biochem. Pharmacol. 57:1059.*
29. Walsh, G. M., L. Annunziato, N. Frossard, K. Knol, S. Levander, J. M. Nicolas, M. Taglialatela, M. D. Tharp, J. P. Tillement, and H. Timmerman. 2001. New insights into the second generation antihistamines. *Drugs 61:207.*
30. Mekori, Y. A., and D. D. Metcalfe. 2000. Mast cells in innate immunity. *Immunol. Rev. 173:131.*
31. Mazzoni, A., H. A. Young, J. H. Spitzer, A. Visintin, and D. M. Segal. 2001. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *J. Clin. Invest. 108:1865.*
32. Caron, G., Y. Delneste, E. Roelandts, C. Duez, N. Herbault, G. Magistrelli, J. Y. Bonnefoy, J. Pestel, and P. Jeannin. 2001. Histamine induces CD86 expression and chemokine production by human immature dendritic cells. *J. Immunol. 166:6000.*
33. Elenkov, I. J., E. Webster, D. A. Papanicolaou, T. A. Fleisher, G. P. Chrousos, and R. L. Wilder. 1998. Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J. Immunol. 161:2586.*
34. Van der Pouw Kraan, T. C., A. Snijders, L. C. Boeije, E. R. de Groot, A. E. Alewijnse, R. Leurs, and L. A. Aarden. 1998. Histamine inhibits the production of interleukin-12 through interaction with H2 receptors. *J. Clin. Invest. 102:1866.*
35. Kay, A. B., L. Barata, Q. Meng, S. R. Durham, and S. Ying. 1997. Eosinophils and eosinophil-associated cytokines in allergic inflammation. *Int. Arch. Allergy Immunol. 113:196.*
36. Nonaka, M., R. Nonaka, K. Woolley, E. Adelroth, K. Miura, Y. Okhawara, M. Glibetic, K. Nakano, P. O'Byrne, J. Dolovich, et al. 1995. Distinct immunohistochemical localization of IL-4 in human inflamed airway tissues: IL-4 is localized to eosinophils in vivo and is released by peripheral blood eosinophils. *J. Immunol. 155:3234.*
37. Moser, R., J. Fehr, and P. L. Bruijnzeel. 1992. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J. Immunol. 149:1432.*
38. Temann, U. A., B. Prasad, M. W. Gallup, C. Basbaum, S. B. Ho, R. A. Flavell, and J. A. Rankin. 1997. A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion. *Am. J. Respir. Cell Mol. Biol. 16:471.*
39. Dabbagh, K., K. Takeyama, H. M. Lee, I. F. Ueki, J. A. Lausier, and J. A. Nadel. 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J. Immunol. 162:6233.*

EXHIBIT B

Ann N Y Acad Sci. 2005 Nov;1056:197-205.

Histamine scavenging attenuates endotoxin-induced acute injury.

Ryffel B, Couillin I, Maillet I, Schnyder B, Paesen GC, Nuttall P, Weston-Davies W.

CNRS Institute Transgenose, IEM, Orleans, France. bryffel@cnrs-orleans.fr

Histamine is an important mediator of early and late inflammatory responses. Here we asked whether scaven endogenous histamine by the arthropod-derived histamine binding protein EV131 diminishes acute respirator syndrome (ARDS) induced by inhaled endotoxin. We demonstrate that EV131 (360 microg given intranasally endotoxin-induced bronchoconstriction and recruitment of neutrophils. Furthermore, EV131 administration dir TNF-alpha and protein leak in the bronchoalveolar lavage fluid. The data suggest that histamine attenuates e induced bronchoconstriction and neutrophil recruitment. Therefore, scavenging of histamine by EV131 may r novel therapeutic strategy in ARDS.

PMID: 16387688 [PubMed - indexed for MEDLINE]